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FOREWORD

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V. Introduction

A. Development of a breast cancer focus in my lab.

This is the first year report for my four year Career Development Award. The purpose of this award was to provide me with additional time and support necessary for me to become versed in the biology of breast epithelial cells and the transformation of these cells into a cancerous state. The Chair of our department agreed to relieve me of my formal teaching responsibilities (which constituted 40% of my effort) once I received the award in order to permit me to focus my efforts on breast cancer research. I was indeed relieved of all of my formal teaching responsibilities. I continued, during the course of the year, to train graduate students. Two graduate students focused their efforts on getting the breast cancer project off the ground. Marvin Nieman has been a Ph.D. student in my lab for 3 years and has been studying the effect of dominant-negative cadherins on squamous epithelial cells. He finished up that project and moved on to the breast cancer project. Ryan Prudoff is a masters student in the lab who spent the past year working with Marvin on a survey of a large number of breast cancer cell lines for expression of cadherins. Dr. Jani Lewis was hired by the department to do my teaching. In addition to her responsibilities as a teacher, Jani will spend time in my laboratory doing research on a project of her choice. In summary, the award of this grant has accomplished its goal which was to provide me with the time to establish a focus in breast cancer research.

B. Introduction to the project.

The formation of a metastatic carcinoma involves not only increased cell growth but also decreased interactions of the tumor cells with one another and with the extracellular matrix. We are particularly interested in the decreased interactions of carcinoma cells with one another. In order for a tumor cell to invade the surrounding tissue, it must down-regulate its interactions with neighboring cells. Down-regulation of these interactions typically results in a change in the morphology of the cell, such that it no longer resembles a typical epithelial cell, but more closely resembles a fibroblastic cell. The cells take on a more scattered appearance with less obvious cell-cell interactions.

Cadherins are cell-surface proteins that play important roles in normal cell-cell interactions. Members of the cadherin family of transmembrane glycoproteins mediate calcium-dependent, homotypic cell-cell adhesion. Numerous studies have implicated E-cadherin as a tumor suppresser protein in carcinomas; i.e., decreased E-cadherin adhesion correlates with a tumor phenotype. The mechanism by which the adhesive function is decreased varies amongst different tumors, but disruption of the function of E-cadherin, regardless of the mechanism, is thought to aid in the formation of a tumor.

Our hypothesis is that an as yet unexplored mechanism of inactivation of E-cadherin function may be operating in some breast tumors. Our idea is that expression of an inappropriate cadherin (for example a mesenchymal cadherin in an epithelial cell) may result in a tumorigenic phenotype. We have previously reported that inappropriate expression of N-cadherin by squamous epithelial cells results in cells that have decreased cell-cell adhesion, are more motile, and have attained a fibroblastic morphology. Each of these properties is characteristic of metastatic tumor cells. Importantly, expression of N-cadherin by the squamous epithelial cells resulted in down-regulation of the normally expressed E-cadherin (11).

In this project we are exploring the possibility that expression of an inappropriate cadherin may be an important factor in breast carcinogenesis. Normal breast epithelial cells express E-cadherin. There have been reports in the literature of breast carcinomas and cell lines established from breast cancers that have lowered expression of E-cadherin or are negative for E-cadherin expression. Our goal is to examine these tissues and cells for the expression of an inappropriate cadherin. The technical objectives we hope to accomplish during the 4 years of this grant are 1) To survey biopsies of breast carcinomas as well as cell lines derived from breast carcinomas for expression of cadherins other than E-cadherin. 2) To determine if the expression of these molecules contributes to the tumorigenic phenotype of cultured breast cancer cells. 3) To explore the mechanisms that

regulate the expression of cadherins in breast epithelial cells. We have made progress on aims 1 and 2 which we present below.

VI. Body

At the end of this section, I have copied the portion of the Statement of Work from the original proposal that is appropriate for the first year of this project and have indicate in red which tasks have been completed.

A. Introduction to the study

E-cadherin expression is down regulated or lost in epithelial tumors from various tissues, including stomach, colon, head and neck, bladder, prostate and breast (1-6). In breast carcinoma, clinical studies have shown that loss of E-cadherin expression correlates with a poor prognosis and an invasive phenotype (6, 7).

In vitro studies support the role of E-cadherin as an invasion suppressor gene. Frixen et al. transfected an invasive E-cadherin-negative breast cell line (MDA-MB-435) with mouse E-cadherin and showed that the transfected cells were less invasive in *in vitro* assays (8). When treated with function blocking E-cadherin antibodies, the transfected cells returned to an invasive phenotype thus implicating E-cadherin as an invasion suppressor. Although a number of studies with breast carcinoma cell lines have shown that loss of E-cadherin generally results in an invasive phenotype, important exceptions have been reported. In one study, two E-cadherin-negative cell lines (SKBr3 and MDA-MB-453) were shown to be noninvasive (9). These authors suggested that in order for E-cadherin-negative cells to be invasive they must also express vimentin.

Hazan et al. reported that expression of N-cadherin by breast carcinoma cells correlated with invasion and suggested that invasion was potentiated by N-cadherin-mediated interactions between the breast cancer cells and stromal cells (10). Data from our laboratory suggests that N-cadherin plays a more direct role in the process of invasion and actually promotes invasion by increasing cell motility when it is expressed by oral squamous cell carcinoma-derived cells (11, Islam, S., Kim, J.-B., Johnson, K.R., and Wheelock, M.J. unpublished).

Thus, the information in the literature concerning the role of cadherins in tumor cell invasion is unclear. This prompted us to revisit the question using new reagents generated by our laboratory to examine previously reported, as well as newly derived, breast cancer cell lines. In the present study we show the following: 1) Decreased expression of E-cadherin does not always correlate with invasion in breast cancer cells; 2) N-cadherin expression does correlate both with invasion and motility in breast cancer cells and likely plays a direct role in promoting invasion; 3) Vimentin expression does not correlate with invasion in cadherin-negative breast cancer cells.

B. Materials and Methods

Cells: Breast carcinoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (SkBr3, MDA-MB-435, MDA-MB-436, BT-549, Hs578t) or minimal essential medium (MEM) with 10% fetal bovine serum (MDA-MB-453). The cell lines MCF-7 and MDA-MB-231 were obtained from Mary Hendrix (University of Iowa, Iowa City, IA) and maintained in DMEM with 10% fetal bovine serum. The cell lines SUM159PT and SUM149 were obtained from the University of Michigan Human Breast Cell/Tissue Bank and Data Base and maintained in Ham's F-12 with 5% fetal bovine serum supplemented with insulin (5 mg/ml) and hydrocortisone (1 mg/ml). The cell line SUM1315m02 was obtained from the University of Michigan Human Breast Cell/Tissue Bank and Data Base and maintained in Ham's F-12 with 5% fetal bovine serum supplemented with insulin (5 mg/ml) and epidermal growth factor (10 ng/ml).

Antibodies and reagents: Unless otherwise stated, all reagents were from Sigma Chemical Co. (St. Louis, MO). The rat monoclonal antibody (E9) against the extracellular domain of human E-cadherin and mouse monoclonal antibodies against β -catenin (5H10) and N-cadherin (13A9) have been previously described (12-14).

Extraction of cells: Monolayers of cells were washed with PBS at room temperature and extracted on ice with 2.5 mls/75 cm² flask 10 mM Tris acetate, pH 8.0, containing 0.5% Nonidet P-40 (BDH Chemicals Ltd., Poole, United Kingdom), 1mM EDTA and 2mM PMSF. The cells were scraped followed by vigorous pipetting for 5 min on ice. Insoluble material was removed by centrifugation at 15,000 g for 10 min at 4° C.

Immunofluorescence: Cells were grown on glass coverslips, fixed with Histochoice (Amresco, Solon, OH), permeablized with methanol for 3 min, washed 3 times with PBS and blocked for 30 min with PBS supplemented with 10% goat serum. Coverslips were exposed to primary antibodies for 1 h, washed 3 times with PBS and exposed to species specific antibodies conjugated to FITC for 1 h. Cells were viewed using a Zeiss Axiophote microscope equipped with the appropriate filters and photographed using Kodak T-MAX 3200 film.

In vitro invasion assays and motility assays: Cells (5×10^5) were plated in the top chamber of Matrigel coated or noncoated transwell membranes (pore size 8 mm; Becton Dickinson, Research Triangle Park, N.C.) for invasion assays or motility assays respectively. In the lower chamber, 3T3 conditioned medium was used as a chemoattractant. The cells were incubated for 24 hours and those which did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transversing the membrane were stained with Diff-Quick (Dade, Aquada, P.R.). Cells in random fields of view at 100x magnification were counted and expressed as the average number of cells/field of view. Three independent experiments were done except where noted.

C. Results

Previous studies showed that, in the majority of cases, E-cadherin-negative breast cancer cells were invasive (9, 15, 16). However, in some cases E-cadherin-negative cells were non-invasive. These studies did not examine the expression of N-cadherin. A recent study from our laboratory showed that expression of N-cadherin by oral squamous cell carcinoma-derived cells resulted in decreased cell-cell adhesion (11). This study also showed that expression of E-cadherin and N-cadherin was mutually exclusive. We therefore hypothesized that for breast cancer cells to be invasive they must not only down-regulate E-cadherin but also upregulate expression of N-cadherin. To test this, we surveyed a large number of cell lines, many of which had been previously characterized, for expression of E-cadherin, N-cadherin and β -catenin.

Table I

Cell Line	E-cadherin	N-cadherin	β -catenin	Vimentin	Invasion
MCF-7	+ ^{ab}	- ^a	+ ^{ade}	- ^b	no ^{ab}
SUM 149	+ ^a	- ^a	+ ^a	- ^a	no ^a
SKBr3	- ^{ab}	- ^a	- ^{ade}	- ^b	no ^{ab}
MDA-MB-453	- ^{ab}	- ^a	- ^{ade}	- ^b	no ^b
SUM 1315m02	- ^a	- ^a	- ^a	- ^a	no ^a
Hs578t	- ^{ab}	+ ^a	+ ^{ade}	+ ^b	yes ^{ab}
BT549	- ^{ab}	+ ^a	+ ^{ade}	+ ^b	yes ^b
MDA-MB-435	- ^{ab}	+ ^a	+ ^{ac}	+ ^f	yes ^c
MDA-MB-436	- ^{ab}	+ ^a	+ ^{ade}	+ ^b	yes ^b
SUM 159PT	- ^a	+ ^a	+ ^a	+ ^a	yes ^a
MDA-MB-231	- ^{ab}	- ^a	+ ^{adc}	- ^b	yes ^b

^a current study; ^b ref (9); ^c ref (8); ^d ref (15); ^e ref (16); ^f Nieman, M.T., Johnson, K.R. and Wheelock, M.J. unpublished observations

The data, which are presented in Table I, supported our hypothesis. The only exception was the cell line MDA-MB-231 which did not express E-cadherin or N-cadherin but, none-the-less, was invasive. This cell line expressed levels of β -catenin that were consistent with expression of a cadherin (see Fig. 1). Therefore, we examined it using degenerate PCR primers designed to amplify all cadherins and found that it expressed cadherin 11, a cadherin that is expressed by some

mesenchymal cells (17). Interestingly, cadherin 11 replaces E-cadherin in cytotrophoblast cells when they invade the uterine wall (18). Thus, we propose that cadherin 11 can act in a manner similar to N-cadherin in promoting cell motility and invasion in breast cancer cells.

Fig. 1 is an immunoblot of representative cell lines presented in Table 1. In the newly derived SUM cell lines we saw examples of each phenotype: SUM149 expressed E-cadherin and β -catenin; SUM159PT expressed N-cadherin and β -catenin; SUM1315m02 did not express E-cadherin, N-cadherin or β -catenin. To compare the newly established SUM cell lines with well characterized breast cancer cell lines, we performed invasion assays on matrigel-coated membranes and motility

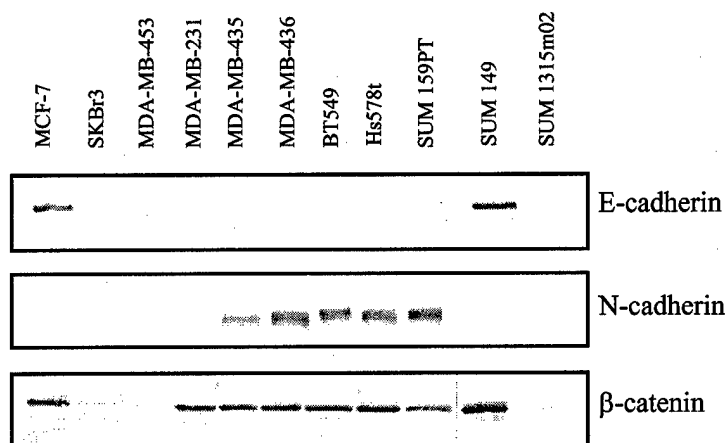


Fig. 1. Cadherin and β -catenin expression in breast carcinoma cell lines. Confluent monolayers of MCF-7, MDA-MB-453, SKBr3, MDA-MB-231, MDA-MB-435, MDA-MB-436, BT-549, Hs578t, SUM159PT, SUM149 or SUM1315m02 were extracted with NP-40. Equal amounts of total protein from each cell extract was resolved by SDS-PAGE, transferred to nitrocellulose and blotted with antibodies against E-cadherin (E9), N-cadherin (13A9) or β -catenin (5H10). Molecular weight markers are shown.

assays on uncoated membranes. Fig. 2 presents data from representative cell lines. We included the previously characterized E-cadherin-positive, non-invasive MCF7 cell line and the N-cadherin-positive, invasive Hs578t cell lines as controls. The N-cadherin expressing cell lines, SUM159PT and Hs578t were substantially more invasive and more motile than the E-cadherin expressing cell lines or the cell lines lacking a cadherin (Fig. 2 and Table 1). Surprisingly, the cell lines that did not express any cadherins, SKBr3 and SUM1315m02, were no more motile or invasive than were the E-cadherin expressing cell lines, MCF-7 and SUM149, suggesting that N-cadherin actively promotes motility and invasion.

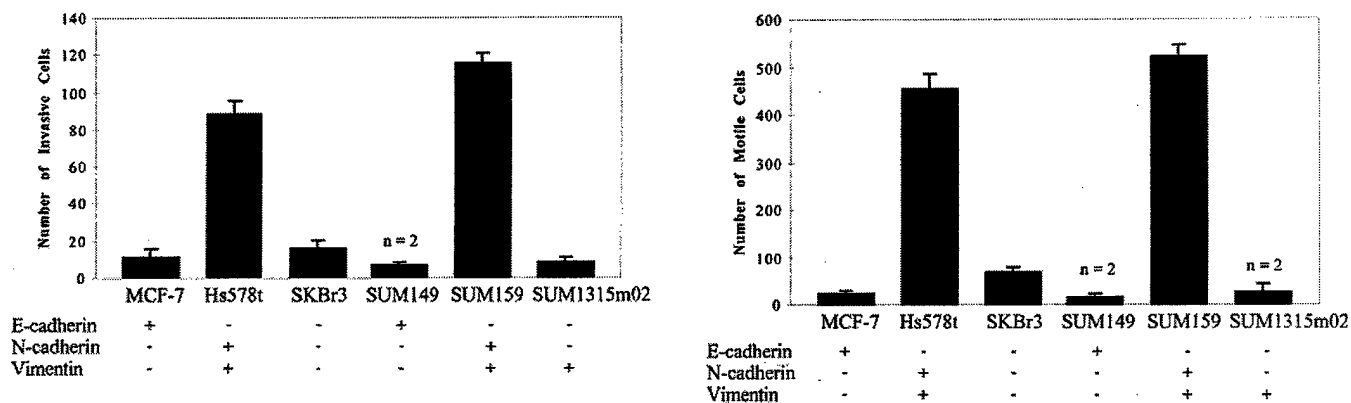


Fig. 2. N-cadherin expression correlates with increased invasiveness and increased motility in breast carcinoma cell lines. Cells (5×10^5) were plated on Matrigel coated or noncoated membranes for invasion assays or motility assays respectively. The cells were incubated for 24 hours and those which did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained and the number transverse the membrane was determined by averaging 10 random fields of view at 100x magnification. The data is expressed as the number of cells/field of view and is the average of three independent experiments except where noted. Error bars indicate standard deviation.

To confirm that the N-cadherin expressed by the breast cancer cell lines was functional, we examined cellular localization using immunofluorescence. Results from two representative cell lines (Hs578t and SUM159) are shown in Fig. 3. Although these cells do not have extensive cell-cell contacts, N-cadherin staining is

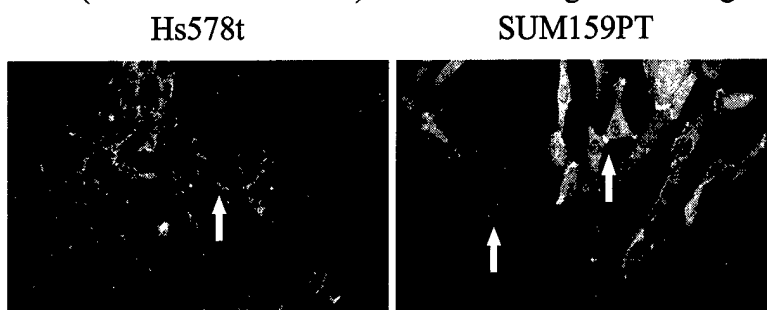


Fig. 3. Localization of N-cadherin in breast cell lines. Cells were grown on glass coverslips and processed for immunofluorescence with a monoclonal antibody against N-cadherin (13A9). Arrows point out regions of cell-cell contact.

localized to the region of cell contact as indicated by an arrow (Fig. 3). Since the cytoplasmic domain of cadherins must interact with the catenins to function in cell adhesion, we used co-immunoprecipitation experiments to show that N-cadherin expressed in each of the cell lines was associated with α -catenin, β -catenin and plakoglobin (data not shown). In addition, the catenins co-localized with N-cadherin in immunofluorescence (data not shown).

Sommers et al. suggested that vimentin expression correlated with an invasive phenotype in E-cadherin-negative breast cancer cell lines. (9, 15). Vimentin is typically expressed by mesenchymal cells which frequently are N-cadherin-positive (14). Since our previous studies had implicated N-cadherin in invasion, and studies by others showed that transfection of vimentin into

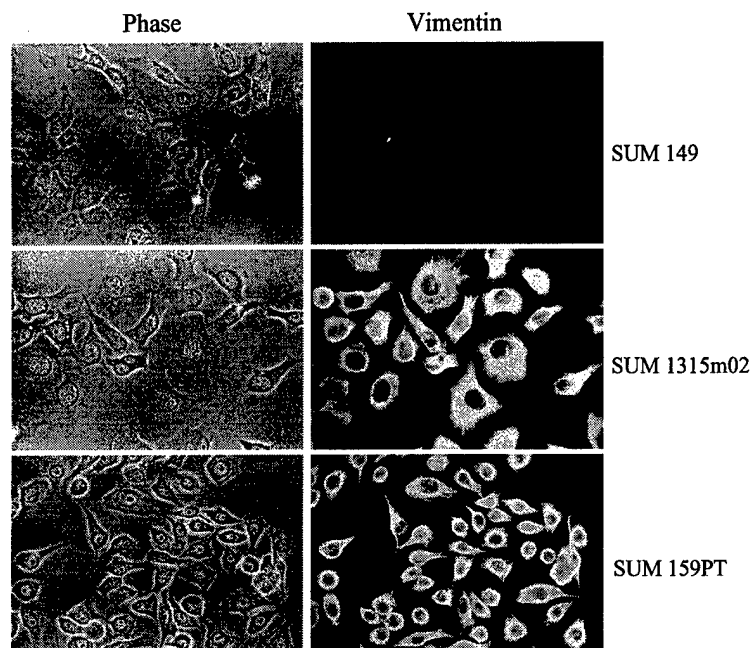


Fig. 4. Morphology and vimentin expression in breast cell lines. Cells were grown on glass coverslips and processed for immunofluorescence with a monoclonal antibody against vimentin (Sigma). Note the extensive network of vimentin filaments in 1315m02, a cell line which is negative for E-cadherin, N-cadherin and b-catenin.

E-cadherin-positive MCF7 cells only slightly increased invasion and did not convert these cells to a malignant phenotype (20), we suspected that the relevant molecule correlating with invasion in the Sommers studies may have been N-cadherin, not vimentin. To determine if our suspicion was correct, we examined several cell lines for expression of E-cadherin, N-cadherin and vimentin and correlated these findings with invasion. SUM1315m02 was an example of a cell line that was E-cadherin-negative, N-cadherin-negative, vimentin-positive and was not invasive (see Fig. 4 and Fig. 1) suggesting that vimentin alone was not sufficient to induce invasion in E-cadherin-negative cells. Fig. 4 also includes an example of noninvasive cells that were negative for vimentin and

positive for E-cadherin (SUM149) and an example of invasive cells that were positive for vimentin and positive for N-cadherin (SUM 159). Each of the N-cadherin-positive cells in this study were also vimentin-positive so we could not examine the invasiveness of vimentin-negative, N-cadherin-positive cells.

VII. Conclusions

In the current study, we have demonstrated that N-cadherin expression in human breast carcinoma cells correlates with an invasive phenotype. Cell lines that did not express any classical cadherins, as evidenced by lack of β -catenin protein, had motility and invasion characteristics similar to cells that expressed E-cadherin. Our hypothesis that loss of E-cadherin alone is not sufficient to

increase invasion in breast carcinoma cells is supported by the observation that function blocking antibodies against E-cadherin did not confer a motile, invasive phenotype on E-cadherin-expressing MCF-7 cells (9). In fact, the current study suggests that expression of N-cadherin, or a similar cadherin such as cadherin 11, may be necessary for increased motility and invasion in breast cancer cells. Hazan et al. suggested that tumor cells expressing N-cadherin may be more invasive due to interactions with N-cadherin-expressing stromal cells (10). Our *in vitro* invasion and motility assays, which do not depend on interactions with stromal cells, suggest that N-cadherin plays a more direct role in cell motility. A recent clinical study suggested that inactivation of E-cadherin is an early event in the progression of lobular breast carcinomas (21). We might suggest here that a subsequent event would be activation of N-cadherin expression.

Previous work suggested that vimentin correlated with an invasive phenotype in breast carcinoma cell lines. Here, we have shown that vimentin alone does not confer an invasive phenotype as the cell line SUM1315m02 expresses vimentin but lacks cadherin expression and is not invasive. Rather, we would suggest that co-expression of vimentin and N-cadherin may correlate with an invasive phenotype in human breast carcinoma cells. We were not able to find an N-cadherin positive cell line that did not express vimentin so we cannot say that N-cadherin expression in the absence vimentin would correlate with invasion. We are currently investigating this idea by attempting to transfect N-cadherin into the vimentin-negative SKBr3 cell line.

At first glance it might seem unlikely that expression of a cell adhesion molecule would confer a motile and invasive phenotype. However, motile cells such as fibroblasts express N-cadherin (14) and a switch from E-cadherin to N-cadherin occurs during gastrulation when epiblast cells ingress through the primitive streak to form the mesoderm (22, 23). In addition, N-cadherin has been shown to correlate with a motile, fibroblastic phenotype in squamous cell carcinomas of the head and neck (11) and in melanomas (24). Thus, it is feasible to hypothesize that an increase in N-cadherin expression would increase the invasive potential of breast carcinoma cells.

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IX. Statement of Work from the original grant proposal

Listed below are the technical objectives for the first year of this proposal. In red I have indicated our accomplishments to date.

Technical objective 1. Survey cell lines and biopsies:

Task 1. Months 1-3: Surveying breast cancer cell lines for E-cadherin expression. This has been accomplished and is presented in Table I and figure 1 above.

Task 2. Months 4-6: Survey E-cadherin negative cell lines for expression of N-cadherin, P-cadherin, R-cadherin and Cadherin 5. This has been accomplished and is presented in Table I and figure 1 above.

Task 3. Months 7-8. Survey frozen histological sections for expression of the cadherin identified in Task 2. Frozen sections have been ordered and will be stained with antibodies against the cadherins as soon as they arrive.

Task 4. Months 9-12. If we do not identify one specific cadherin in task 2 we will perform PCR using degenerate primers to identify the cadherin of interest. This is irrelevant at this point as we have identified N-cadherin as expressed by invasive breast carcinoma cells. In addition, we have identified a new, previously unreported cadherin that shares some homology with cadherin 11. The characterization of this cadherin will be a component of the next funding period.

At the end of year 1, we expect to have identified an inappropriate cadherin that is expressed in breast tumors. As noted above, we have accomplished this goal.

X. Publication of the results of this study

The data discussed above was presented at the 89th annual meeting of The American Association for Cancer Research in the form of a poster entitled "**N-cadherin expression promotes invasive phenotype in breast carcinoma cell lines**" by Nieman, M.T., Ethier, S.P., Johnson, K.R., and Wheelock, M.J. The data will be submitted as a full paper once we have completed an experiment to show that N-cadherin is indeed responsible for the altered phenotype of these cells.

XI. Awards

My student was selected to receive a travel award to attend the 89th meeting of the American Association for Cancer Research in order to present this work.

XII. Appendix

Below is the abstract of this work that was presented at the 89th meeting of the American Association for Cancer Research.

N-cadherin expression promotes invasive phenotype in breast carcinoma cell lines. Nieman, M.T.¹, Ethier, S.P.², Johnson, K.R.¹, Wheelock, M.J.¹ ¹The University of Toledo, Department of Biology, Toledo, OH 43606, ²The University of Michigan Medical School, Department of Radiation Oncology, Ann Arbor, MI 48109

The E-cadherin/catenin complex is important in maintaining an epithelial phenotype and normal tissue architecture. E-cadherin expression is decreased or absent in invasive epithelial tumors from various tissues. This has lead to the hypothesis that down regulation of E-cadherin allows a cell to disassociate from the surrounding cells and to invade the surrounding tissues. In vitro data has shown that not all E-cadherin negative breast carcinoma cell lines are invasive. In the present study, we examine the expression of N-cadherin in breast carcinoma cell lines. The E-cadherin negative cell lines that have previously been described as invasive (Hs578t, MDA-MB-435, MDA-MB-436 and BT-549) as well as a new invasive cell line (SUM159) expressed N-cadherin. In contrast, E-cadherin negative cell lines which were not invasive (SKBr-3, MDA-MB-453 and SUM1315m02) did not express N-cadherin. The N-cadherin in these breast cell lines is functional in that it forms a complex with α -catenin and β -catenin as determined by coimmunoprecipitation experiments. These observations suggest that the loss of E-cadherin is not sufficient to confer an invasive phenotype in breast carcinoma cell lines and that the expression of a mesenchymal cadherin (N-cadherin) in breast epithelial cells promotes invasiveness.